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DRAWINGS ATTACHED.

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COMPLETE SPECIFICATION.

Preparation of a New Antibiotic Aspartocin.

We, AMERICAN CYANAMID COMPANY, a corporation organized under the laws of the State of Maine, United States of America, of 30 Rockefeller Plaza, New York 20, State of New York, United States of America, do hereby declare this invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement :—

This invention relates to a new antibiotic designated aspartocin, and to salts thereof. The invention also relates to the production of aspartocin by fermentation, to methods for its recovery and concentration from crude solutions thereof, to processes for its purification and to methods for the preparation of its salts.

The present invention includes within its scope the antibiotic in dilute forms, as crude concentrates, and in pure crystalline forms. These novel products are active against a variety of microorganisms including gram-positive bacteria. The effects of the new antibiotic on specific microorganisms together with the chemical and physical properties of the antibiotic differentiate it from previously described antibiotics.

In accordance with the invention a process for the production of the new antibiotic aspartocin which comprises cultivating an antibiotic aspartocin producing strain of *Streptomyces griseus* var. *spiralis* or *Streptomyces violaceus* in an aqueous nutrient medium containing assimilable sources of carbon, nitrogen and inorganic salts under submerged aerobic conditions.

[*Price 4s. 6d.*]

The following is a general description of the organism *S. griseus* var. *spiralis* based on the diagnostic characteristics observed. The underscored descriptive colors are those of Ridgeway—"Color Standards and Color Nomenclature."

Amount of Growth—Growth moderate to good on many media, spreading on starch-containing media; poorer, restricted growth on certain synthetic media.

Aerial Mycelium and/or spore colour—Spores *en masse* are *Pale Olive—Buff*; non-sporing aerial mycelium colourless to whitish.

Soluble Pigments—None.

Reverse Colour—In shades of buff to yellowish on most media.

Miscellaneous Physiological Reactions—No growth on cellulose; complete liquefaction of gelatin; formation of acid curd and clearing of purple milk; and negative H_2S reaction on peptone-iron agar media.

Morphology—Sporiferous appendages arise from aerial mycelium as coils of a few turns or short spirals. Spores elongate, rod - like, truncate, ($1.0—1.2\mu \times 0.6\mu$) spaced regularly apart in chains.

Temperature affect—Optimal range for growth and sporulation 18° C. to 37° C. Maximum temperature for growth 42° C., no growth at 48° C. Minimum temperature 10° C., no growth at 4° C.

The cultural characteristics of the new variety of *S. griseus* are set forth in the following table. The underscored descriptive colours were taken from Ridgeway.

TABLE 1.

Streptomyces griseus var. *spiralis.*

Medium.	Amount of Growth.	Aerial Mycelium and Spore Colour.	Soluble Pigment.	Reverse Colour.	Remarks.
Waksman's Starch Agar	Good ; spreading	*Pale Olive-Buff* ; Sporulation heavy	None	*Deep Colonial Buff*	Moderate starch
Asparagine dextrose meat extract agar	Moderate	*Pale Olive-Buff* Sporulation moderate	None	*Colonial Buff*	Limited colourless exudate
Acid asparagine dextrose meat extract agar	Moderate restricted	*Pale Olive-Buff* ; Sporulation very slight	None	*Colonial Buff*	
Synthetic Agar (Czapek's Agar)	Thin ; restricted	White powdery aerial mycelium ; feathery margins	None	White	
Emerson's Agar	Moderate	*Pale Olive-Buff* ; Sporulation light ; colonies lightly rimose	None	*Colonial Buff*	
Nutrient Agar	Poor, thin growth	None	None	Colourless	
Calcium malate Agar	Moderate ; restricted	*Pale Olive-Buff* ; Sporulation light			Moderate zone of malate clearing
Cobalt Amidex Agar	God ; spreading	*Olive-Buff to Pale Olive-Buff* ; Sporulation heavy	None	*Honey Yellow*	Lightly zonate
Potato dextrose Agar	Moderate	*Pale Olive-Buff* ; Sporulation heavy	None	*Colonial Buff*	

TABLE 1—*continued.*

Streptomyces griseus var. *spiralis.*

Medium.	Amount of Growth.	Aerial Mycelium and Spore Colour.	Soluble Pigment.	Reverse Colour.	Remarks.
Bennett's Agar	Moderate	*Pale Olive-Buff*; Sporulation heavy	None	*Honey Yellow*	Limited colourless exudate
Corn steep Liquor Agar	Moderate	*Pale Olive-Buff*; Sporulation moderate	None	*Cream Colour*	
Sabouraud's maltose	Moderate	Aerial mycelium white; no sporulation; central colony zones barren	None	*Ochraceous-Buff*	
Yeast-Malt Agar	Moderate	*Pale Olive-Buff*; Sporulation heavy	None	*Ochraceous-Buff*	Moderate colourless

In gross appearance this new strain bears close resemblance to several strains of *S. griseus*. Spore coloration, reverse colours and growth habits are similar. However, when the sporophores are compared microscopically, all of the *S. griseus* strains have straight to flexuous chains of globose to elliptical spores, in contrast to the coiled and spiralled chains of truncate, rod-like spores of the new strain. The combination of helicoidal sporophores and bacillary-type spores justifies varietal status for this strain in the *S. griseus* complex. The name *S. griseus* var. *spiralis* has been chosen to be descriptive of the sporiferous structures of the organism. The new isolate, when keyed according to Waksman and Lachevalier, "Actinomycetes and Their Antibiotics," falls into subdivision III—no soluble pigment in organic media—and fits best into the category (g)—growth colourless to yellowish to olive-buff—in which *S. griseus* is the representative species. A viable culture of *S. griseus* var. *spiralis* has been deposited at the American Type Culture Collection in Washington, D.C., where it has been assigned the accession number ATCC 13733.

Additionally, it has been found that aspartocin can also be produced by a species of *Streptomyces* which we have designated *Streptomyces violaceus*.

The following is a general description of the organism *Streptomyces violaceus* based on the diagnostic characteristics observed. The underscored descriptive colours were taken from Ridgway.

Amount of Growth—Moderate to good growth on most media; spreading on growth on most media; spreading on Waksman's Starch, Czapek's, Corn Steep Liquor, and Cobalt-amidex agars.

Aerial Mycelium and/or Spore Colour—Spores *en masse* Light Mouse Gray on most media which support sporulation.

Soluble Pigment—In reddish to vinaceous to bluish shades on media which permit pigment formation.

Reverse Colour—In reddish to vinaceous to bluish or even brownish shades, depending upon the medium.

Miscellaneous Physiological Reactions—Moderate starch hydrolysis; no H_2S produced; moderate gelatin liquefaction; cellulose decomposed.

Morphology—Sporiferous appendages arising as coils or loose spirals from aerial hyphae. Spores smooth, typically globose, but with occasional elliptical ones in chain, 1.0—1.2μ.

Temperature Relations—Growth fair at 24—28° C.; good at 32—37° C.

The cultural, physiological and morphological characteristics of the organism *Streptomyces violaceus* are set forth in the following tables. The underscored descriptive colours were taken from Ridgway.

TABLE 2.

Characteristics of Streptomyces Violaceus when Grown on Several Differential Agar Media in Petri Dishes,

Medium.	Amount of Growth.	Aerial Mycelium and Spore Colour.	Soluble Pigment.	Reverse Colour.	Remarks.
Waksman's Starch Agar	Good; spreading Broadly	Spores *Light Mouse Gray*; dark non-sporing sectors prominent; sporulation moderate	None	*Dusky Drab*	Moderate Starch hydrolysis
Asparagine dextrose meat extract agar	Moderate	Spores *Light Mouse Gray*; sporulation thin	None	Light brownish with *Vinaceous-Gray* patches	Faintly zonate; margins thin, submerged
Acid asparagine dextrose meat extract agar	Poor; restricted	None	None	Colourless	
Synthetic Agar (Czapek's Agar)	Good; spreading	Spores *Light Mouse Gray*; moderate sporulation with sectoring	Bluish-purple; moderate	*Anthracene purple*	
Emerson's Agar	Good	Spores *Light Mouse Gray*; sporulation heavy	Reddish; very light	*Deep Mouse Gray*	
Nutrient Agar	Moderate	Trace of whitish to grayish aerial growth	None	Colourless to brownish	
Calcium malate	Moderate	Very poor sporulation; aerial growth *Pale Mouse Gray*	None	*Vinaceous-Fawn*	Large zone of malate clearing
Yeast extract—Malt extract agar	Moderate	Spores *Light Mouse Gray*; sporulation moderate	None	*Fuscous to Bone Brown*	Thin submerged margins

TABLE 2—*continued.*

Characteristics of Streptomyces Violaceus when Grown on Several Differential Agar Media in Petri Dishes.

Medium	Amount of Growth	Aerial Mycelium and Spore Colour	Soluble Pigment.	Reverse Colour.	Remarks.
Waksman's Glucose Agar	Moderate	Spores *Pale Mouse Gray*; sporulation light	*Vinaceous* moderate	*Deep Brownish Drab*	Marginal zones wrinkled and without aerial growth
Krainsky's dextrose Agar	Moderate	Spores *Pale Mouse Gray*; sporulation very light	None	*Pinkish Buff* to *Fawn* colour	
Potato dextrose Agar	Moderate	Aerial growth scanty; sporulation *Light Mouse Gray*, sparse	Pinkish-vinaceous light	*Vinaceous-slate* to *Deep Slaty Brown*	Thin submerged margins
Bennett's Agar	Good	Spores *Light Mouse Gray*; sporulation moderate	None	*Deep Mouse Gray*	Thin submerged margins
Corn Steep liquor Agar	Moderate; thin spreading	Sporulation *Light Mouse Gray* to *Light Drab*; sporulation moderate	None	*Fawn* colour	Thin submerged margins
Sabouraud's maltose Agar	Good	Spores *Light Mouse Gray*; sporulation moderate	None	*Orange cinnamon*	
Cobalt-amidex Agar	Moderate; spreading	*Light Mouse Gray*	Reddish; light	*Vinaceous-Slate* to *Deep Slaty Brown*	Margin thin, submerged
Czapek's-Dox Mannitol Agar	Good; spreading	Aerial mycelium white with spores *Pale Mouse Gray*; sporulation moderate	Vinaceous; light	*Vinaceous-Slate*	

TABLE 3.

Observations of Some Miscellaneous Physiological Tests on *Streptomyces violaceus*.

Medium	Amount of Growth	Aerial Mycelium and Spore Color	Soluble Pigment	Reverse Colour	Remarks
Peptone-Iron Agar	Good	—	—	—	Negative H_2S Reaction
Gelatin	Moderate	None	None	—	Moderate liquefaction; part of tube not liquefied
Potato Plugs	Good; covering entire exposed surface	*Light Mouse Gray* in sporulating areas	Reddish; light		Plug slightly darkened
Carrot Plugs	Moderate; covering entire exposed surface	*Light Mouse Grey* in sporulating areas	None		
Litmus Milk	Moderate	Clearing of Purple milk, and curd precipitated; *p*H 7.0			
* Cellulose (filter paper in Czapek's solution)	Moderate	None	Vinaceous light		Filter paper decomposed in growth areas

* Incubation 21 days.

TABLE 4.

Morphological Features of *Streptomyces violaceus*.
MEDIUM : Waksman's Starch Agar.

Culture No.	Aerial Mycelium	Spore Shape	Spore Size	Remarks.
Streptomyces violaceus	Sporiferous appendages arising as coils or loose spirals from aerial hyphae	Typically globose, but with occasional elliptical spore in chains	1.0—1.2μ	Spores, when viewed under the electron microscope, had smooth walls

A viable culture of *Streptomyces violaceus* has been deposited with the American Type Culture Collection in Washington, D.C., where it has been assigned ATCC accession number 13734.

It is to be understood that for the production of the antibiotic of this invention, the present invention is not limited to these two organisms fully answering the above growth and microscopic characteristics. In fact, it is desired and intended to include the use of any antibiotic aspartocin producing mutants obtained from the described organism by various means, such as X-radiation, ultraviolet radiation, nitrogen mustard or phage exposure.

The cultivation of the new strain of *S. griseus* var. *spiralis*, as well as of *S. violaceus*, may take place in a variety of liquid culture media. Media which are useful for the production of aspartocin include an assimilable source of carbon such as starch, sugar, molasses, or glycerol, an assimilable source of nitrogen such as protein, protein hydrolysate, polypeptides, amino acids, or corn steep liquor, and inorganic anions and cations, such as potassium, sodium, calcium, sulphate, phosphate, or chloride. Trace elements such as boron, molybdenum and copper are supplied as needed in the form of impurities by other constituents of the media. Aeration in tanks and bottles is provided by forcing sterile air through or onto the surface of the fermenting medium. Further agitation is provided in tanks by a mechanical impeller. An antifoaming agent such as 1% octadecanol in lard oil may be added as needed.

For shaker flask fermentations, 100 millilitre portions of the following liquid inoculum in 500 millilitre flasks are inoculated with an agar slant of the culture.

Liquid Inoculum

Soy bean meal	20 grams per litre
Starch	80 grams per litre
Corn steep liquor	5 grams per litre
Calcium carbonate	3 grams per litre

The flasks are incubated on a reciprocating shaker and agitated vigorously for 48 hours.

For the production of the antibiotic in tank fermenters, the following fermentation medium is preferably used.

Fermentation Medium

Molasses	20 grams per litre
Corn Starch	10 grams per litre
Bactopeptone	10 grams per litre
Calcium carbonate	1 gram per litre

Each tank is inoculated with 1% of a culture broth fermentated as described above for shaker flask fermentation. Aeration is supplied at the rate of 0.2—2.0 volumes of sterile air per volume of broth per minute and the broth is agitated by an impeller driven at about 120—160 r.p.m. The temperature is maintained at 20—35° C., usually at 28° C. The fermentation may be continued for from 24—240 hours, at which time the activity is harvested.

After the fermentation is completed, the culture broth containing the antibiotic of this invention is filtered at preferably *p*H 5.0 to remove the mycelium from the broth. Diatomaceous earth or any of the conventional filtration aids may be used to assist the filtration which is carried out using standard equipment. Thereafter, aspartocin may be recovered from the mycelial cake by extraction procedures as described below.

Aspartocin may be extracted from the mycetral cake with a first solvent at *p*H 1—2 and/or 9—10 then precipitated or solvent extracted using a second immiscible or partially immiscible solvent, such as butanol at *p*H 1—3 when the first solvent is aqueous. Roughly, 80—90% of the activity is contained in the mycelial cake.

When the butanol solution is concentrated ($^1/_{25}$ to $^1/_{50}$ of its volume) to anhydrous butanol, the antibiotic is precipitated. The butanol-precipitate mixture is stirred with acetone or petroleum ether and the precipitate removed by centrifugation. The antibiotic is then washed with acetone, and dried yielding an amorphous product.

Alternatively, calcium chloride or other alkalin earth salts may be added to produce

the corresponding salt of aspartocin and facilitate precipitation and/or crystallization thereof.

Purification of aspartocin may be obtained by crystallization of calcium aspartocin from solvent mixtures such as butanol-water or methanol-water, in a standard manner.

The novel antibiotic aspartocin of this invention is composed of the elements carbon, hydrogen, nitrogen, sulphur and oxygen. Elemental analyses of aspartocin show values in substantially the following proportions by weight:

Carbon	53.58
Hydrogen	7.58
Nitrogen	13.58
Sulphur	0.36
Oxygen (by difference) ..	24.90

The product has an optical rotation of $[\alpha]_D^{25°} = +26.4°$ (c, 2.1% solution in methanol). The product shows no characteristic ultraviolet absorption. The product is soluble in water below *p*H 3.0 and above *p*H 3.6. The isoelectric point is near 3.3. The free acid of aspartocin is soluble in methanol, ethanol, butanol, glacial acetic acid, water and wet butanol. It is soluble to the extent of 0.6 mg./ml. in acetone and 0.1 mg./ml. in ethyl acetate and ether.

An infra-red absorption spectrum of the free acid of aspartocins was prepared in a standard manner by mixing with crystals of KBr and pressing into a disc. The compound exhibits characteristic absorption in the infra-red region of the spectrum at the following wavelengths expressed in microns: 3.07, 3.45, 6.03, 6.53, 6.89, 7.15, 8.10 and 9.82. The infra-red curve is shown in the accompanying drawing.

Microbiological and paper chromatographic analyses of aspartocin acid hydrolysates indicate the following amino acid contents:

	%	Molar Ratio
D or L-Aspartic Acid	35	4
L-Proline ..	8	1
L-Valine	8	1
Glyc		1
Glutamic acid ..	10	1

Aspartocin shows the following R*f* values in the solvent systems indicated below:

R*f* Value	*Solvent System*
0.25	5% NH_4Cl
0.38	*s*-collidine saturated with water.
0.14	0-2M phosphate *p*H 6.0
0.01	100 *n*-amyl acetate 30 dibutyl ether 5 acetal acid 50 water
0.90	100 chloroform 35 90% phenol 4 acetic acid 2 pyridine 50 water
0.01	200 benzene 100 cyclohexane 15 acetic acid 50 water
0.95	200 *m*-cresol 1 HOAc 1 pyridine 100 water
0.05	200 *n*-butanol 50 pyridine 200 water
0.30	200 *m*-cresol 100 0.1M phosphate *p*H 7.0 5% NaCl
0.48	200 *m*-cresol 100 0.1M phosphate *p*H 6.0
0.80	200 *n*-butanol 50 pyridine 50 acetic acid 100 water
0.01	Water adjusted to *p*H 2.3 with tartaric acid
0.01	200 *n*-butanol 100% $NaHCO_3$
0.01	100 1,2-dichloroethane 100 carbon tetrachloride 25 acetic acid 50 water
0.01	100 $CHCl_3$ 40 pyridine 40 HOAc 50 water
0.97	200 90% phenol 50 *m*-cresol 8 HOAc 8 pyrdine 50 water

Aspartocin is clearly distinguished from other antibiotics by its chemical and

physical properties as evidenced by those described above, by its antimicrobial spectrum and by paper chromatography. The specific antimicrobial activity of the antibiotic of this invention is presented in the table below which shows the concentration of aspartocin required to inhibit the growth of representative microorganisms in trypticase soy broth :

TABLE 2.

Organism	Minimum Inhibitory Concentration, mcg./ml.
Staphylococcus aureus ATCC 6538	15.5
Staphylococcus aureus 209P	15.5
Streptococcus pyogenes C-203	2.0
Streptococcus pyogenes NY-5	4.0
Cornynebacterium xerosis NRRL B-1397	1.0
Bacillus cereus	4.0
Sarcina lutea	2.0
Bacillus polymyxa	4.0
Bacillus megatherium	0.25
Erysipelothrix rhusophthiae	0.5
Bacillus subtilis ATCC 6633	4.0
Klebsiella pneumoniae	>250
Pasteurella multocida	>250
Salmonella gallinarum	>250
Escherichia coli	>250
Proteus vulgaris	>250
Candida albicans	>250
Mycobacterium ranae	62.0
Mycobacterium 607	62.0

Aspartocin is highly active *in vitro* against Staphylococci tested including *S. aureus* and *S. albus*, coagulase positives and negatives. Streptococci including à-haemolytic, β-haemolytic and non-haemolytic strains are sensitive to the new antibiotic. Many of the above-mentioned organisms are isolates obtained from clinical sources and are to some extent resistant to either penicillin or the tetracycline antibiotics. Aspartocin is also highly active subcutaneously against the standardized infections in mice, *Streptococcus* C203, *Diplococcus pneumoniae* SV1 and *Staphylococcus aureus*.

Aspartocin has also been found to be substantive from aqueous or organic solution to cloth such as cotton fabric and may be added to cotton cloth for the purpose of rendering the cloth bacteriostatic in substantially the same manner as has been found useful with the antibiotic neomycin.

Aspartocin is especially valuable because of its growth promoting properties in fowl, i.e. chickens. The antibiotic may be added to a purified chick diet containing casein as the protein source and sucrose as the carbohydrate source supplied with all of the known vitamins and minerals. It has been found that aspartocin may be usefully added to such diets in amounts as little as 5 or 10 parts per million per kilogram of diet. More may be added if desired but so far no need has been found for adding more than 500 milligrams per kilogram of diet. The per cent increase in weight of the chickens over the controls ranges from about 25% to 30%. Aspartocin has not as yet been demonstrated to be useful in human therapy.

The invention will be described in greater detail in conjunction with the following specific examples.

EXAMPLE 1.

Inoculum Preparation.

A typical medium used to grow the primary inoculum is prepared according to the following formula :

Soy bean meal	20 grams
Starch	20 grams
Corn steep liquor	5 grams
Calcium carbonate	3 grams

Water to 1,000 millilitres.

A yeast-malt agar slant of a culture of the species *S. griseus* var. *spiralis* is incubated for a week. At this time the spores and mycelium are transferred to two 500 millilitre flasks which contain 100 millilitres of the above medium. The flasks are placed on a reciprocating shaker and agitated vigorously for 48 hours at 28° C. The flask inocula are transferred to 9 litre bottles which contain 6 litres of the above liquid medium. These bottles are aerated for 24 hours to encourage further growth. At the end of this time the 9 litre bottles are used to seed fermenter tanks.

EXAMPLE 2.

Fermentation.

A fermentation medium is prepared according to the following formula :

Molasses	20 grams
Corn starch	10 grams
Bactopeptone	10 grams
Calcium carbonate	1 gram

Water to 1,000 millilitres.

The fermentation medium is sterilized at 120° C. with steam at 15 pounds pressure for 60 minutes. The *p*H of the medium before and after sterilization is 6.75. 1500 litres of the sterile medium in 1000 gallon fermenters are inoculated with 12 litres of the bottle inoculum described above and the fermentation is carried out at 28° C. for 90 hours. The medium is agitated by an impeller operating at 100 revolutions per minute. At the end of the fermentation the mash is assayed.

EXAMPLE 3.

Isolation.

Two hundred litres of fermented mash are mixed with 6000 grams of diatomaceous earth, adjusted to *p*H 5.0, filtered and the filtrate discarded. The mycetral cake is washed twice—first with 40 litres of H_2O at *p*H 5.0 and then with 40 litres of acetone. Both washes are discarded. The mycelial cake is extracted twice with 50 litres of H_2O at *p*H 2.0 adjusted with H_2SO_4, and once with 45 litres of H_2O at *p*H 9.5 adjusted with NaOH. The acid H_2O and alkaline H_2O extracts are each extracted with $\frac{1}{2}$ volume of *n*-butanol at *p*H 2—3. The separated butanol extracts are adjusted to *p*H 5—6 and concentrated to $^1/_{25}$ to $^1/_{50}$ of their volume causing the antibiotic to precipitate. The butanol concentrate of the acid H_2O extract is stirred with three volumes of acetone, and the precipitate removed by centrifugation, washed with acetone and dried. The yield of product is 151 grams. The butanol concentrate of the alkaline H_2O is stirred with three volumes of petroleum ether, the precipitate centrifuged off, washed with acetone and dried. The yield of product is 41 grams.

EXAMPLE 4.

Isolation of Calcium Aspartocin.

Ten grams of calcium chloride and 300 grams of diatomaceous earth are added to 10 litres of fermented broth. The mixture is stirred, adjusted to *p*H 5.0 and maintained at this *p*H for 10 minutes during stirring. The precipitated calcium aspartocin is filtered off with the mycelial cake and the spent filtrate discarded. The mycelial cake is washed by stirring in 2 litres of H_2O at *p*H 5.0. After filtration, the inactive H_2O wash is discarded. The mycelial cake is further washed by stirring with 2 litres of acetone. The mycelial cake is filtered and the inactive acetone wash discarded. The mycelial cake is extracted twice by stirring 10 minutes with 2 litres of H_2O at *p*H 1.0 adjusted with HCl. The antibiotic enriched acid H_2O is extracted with $\frac{1}{2}$ volume of *n*-butanol. The butanol extract is adjusted to *p*H 5.0 and concentrated under reduced pressure with the addition of H_2O to maintain a wet butanol solution. The solution is concentrated to approximately 500 millitres and a wet butanolic solution of $CaCl_2$ is added at *p*H 5.0 until there is no further precipitation. The precipitated calcium aspartocin is removed by centrifugation, washed with wet butanol, followed by an acetone wash and dried. The yield of crude crystalline product is 6 grams. This product assays approximately 90% pure.

EXAMPLE 5.

Crystallization of Calcium Aspartocin.

Forty grams of product prepared according to the procedure of Example 3, is dissolved in 200 millilitres of H_2O. An aqueous solution of 40 grams of $CaCl_2$ is added and the solution adjusted to *p*H 9.0 with NaOH and filtered. The filtered solution is readjusted to *p*H 5.0 with HCl and the precipitated calcium aspartocin is removed by centrifugation. The precipitate is dissolved in 400 millilitres of H_2O at *p*H 2.5 and 200 millilitres of methanol added. The solution is filtered and the methanol-H_2O solution adjusted to *p*H 5.0 and stored at 5° C. The crystalline calcium aspartocin is removed by centrifugation. The crystalline product is dissolved in 200 millilitres of methanol at *p*H 3.0 adjusted with HCl. The solution is stirred with 4 grams of carbon and filtered. The filtered solution is adjusted to *p*H 5.0 using NH_4OH. An immediate crystalline precipitate is formed which is removed by filtration washed with methanol and dried. The first crop of crystalline calcium aspartocin yields 3.85 grams. The mother liquor of the first crop of crystals is stored at 5° C. for 16 hours. A second crop of crystalline calcium aspartocin is removed by filtration, washed with methanol and acetone and dried. The second crop yields 3.37 grams. Additional crops are recovered by concentrating the mother liquor under reduced pressure and storing at 5° C.

EXAMPLE 6.

Preparation of the Free Acid of Aspartocin.

One gram of calcium aspartocin similar to that prepared according to the procedure of Example 5 is dissolved in 25 millilitres of H_2O by adjusting with HCl to *p*H 1.5. The aqueous solution is adjusted to *p*H 3.0 and mixed with 15 millilitres of an aqueous solution saturated with NaCl. The precipitated free acid is washed freely with H_2O. The precipitate is dissolved in 50 millilitres of methanol, filtered and concentrated to approximately 10 millilitres. Forty millilitres of H_2O is added to the methanol solution precipitating the free acid. The precipitate is washed with H_2O, followed by an acetone wash, removed by centrifugation and dried. Yield of free acid of aspartocin is 340 milligrams. The chemical analysis of this product and its other chemical, physical and biological properties have already been described.

EXAMPLE 7.

Preparation of the Picrate Salt of Aspartocin.

Ten grams of product prepared according to the procedure of Example 3 is dissolved in 250 millilitres of H_2O at *p*H 2.0. An

aqueous solution saturated with picric acid is added until no further precipitate forms. The precipitate is removed by centrifugation and washed with H_2O. The picrate is dissolved in 200 millilitres of acetone, concentrated under reduced pressure, to about 100 millilitres, and the picrate precipitated by the addition of H_2O until precipitation is complete. The supernatant is decanted and the picrate dissolved in 200 millilitres of acetone. Butanol is added, and the solution concentrated under reduced pressure to approximately 100 millilitres of an anhydrous butanol solution. The butanol solution is mixed with three volumes of ether, the precipitated picrate removed by centrifugation, washed with ether and dried. The yield of picrate is 8.5 grams.

EXAMPLE 8.

Preparation of Sodium and Potassium Salts of Aspartocin.

One hundred grams of calcium aspartocin similar to that described in Example 4, is dissolved in 1 litre of H_2O at *p*H 2.0 adjusted with HCl. The aqueous solution is adjusted to *p*H 3.3 and extracted 2 times with approximately 1 litre of butanol each time. The butanol extract after washing with H_2O at *p*H 3.4, is dried over Na_2SO_4 and filtered.

A $\frac{1}{3}$ portion, 777 millilitres, of the butanol solution is stirred with 200 millilitres of H_2O, adjusted with NaOH to *p*H 8.5 and concentrated to approximately 200 millilitres of an anhydrous butanol solution. A crystalline sodium salt which precipitates is removed by centrifugation, washed with butanol, ethanol, acetone and dried. The yield of crystalline sodium salt, is 3.0 grams. A second crop of sodium salt is collected by stirring the mother liquor with 10 volumes of acetone and removing the precipitate by centrifugation. The precipitate is washed with acetone and dried to yield 13.1 grams of material.

A potassium salt is prepared from a second $\frac{1}{3}$ portion of the butanol solution by the same procedure except that KOH is used to adjust to *p*H 8.5.

Five hundred milligrams of the free acid aspartocin is dissolved in 5 millilitres of ethanol. A saturated ethanolic solution of KOH is added dropwise to the ethanol solution of aspartocin until no further precipitate results. The precipitated potassium salt is removed by centrifugation, washed with 8 millilitres of ethanol and 10 millilitres of acetone. The salt is dried under vacuum to yield 220 milligrams.

The sodium salt is prepared as described above using the same concentration of free acid and adding a saturated ethanolic solution of NaOH to form the salt. The yield of dried sodium salt is 185 milligrams.

EXAMPLE 9.

Production of Aspartocin by S. violaceus.

The conditions of inoculum preparation and fermentation are the same as previously described in Examples 1 and 2 of this application. The culture is fermented in two tanks using 100 litres of fermentation medium in each tank.

Tank 1 Fermentation Medium	Grams per litre	Tank 2 Fermentation Medium.	Grams per litre
Peptone	25.0	Soy Bean Meal	40.0
Corn Starch	10.0	Corn Starch	10.0
Molasses	20.0	Molasses	20.0
Lactalbumin	5.0	$CaCO_3$	3.0
$Mg_2SO_4.7H_2O$	5.0		
$CaCO_3$	1.0		

After 137 hours of fermentation, the tank mashes are harvested, pooled, and processed as follows :

Isolation and Purification of Aspartocin from S. violaceus.

190 Grams of calcium chloride and 5700 grams of diatomaceous earth are added to 190 litres of fermented mash. The mixture is adjusted to *p*H 5.5, stirred for 20 minutes and filtered. The precipitated antibiotic is filtered off with the mycelial cake and the spent filtrate discarded. The mycelial cake is washed with 60 litres of water at *p*H 5.0—5.5 and filtered. The inactive water wash is discarded. The mycelial cake is extracted twice by stirring 20 minutes with 60 litres of water at *p*H 9.8—10.0. After filtration the alkaline water extracts are pooled, adjusted to *p*H 1.0—3.0 with HCl and extracted twice with $\frac{1}{4}$ volume of *n*-butanol. The 65 litres of pooled butanol extract are adjusted to *p*H 5.0—7.0 and concentrated under reduced pressure to approximately

4 litres of anhydrous butanol. The antibiotic precipitates and is removed by centrifugation and the butanol supernatant discarded. The precipitate is dissolved in approximately 500 millilitres of water at pH 1.5 and the solution is filtered. The aqueous solution is extracted 3 times with 300 millilitre portions of *n*-butanol. The butanol extract 950 millilitres, is adjusted to *p*H 5.0 to 5.5 and stirred with 50 millilitres of a wet butanol solution containing 3 grams of calcium chloride at a *p*H of 5.0—5.5. The precipitated calcium aspartocin is removed by centrifugation, washed with wet butanol, washed again with acetone and dried. The yield of product is 7.2 grams.

WHAT WE CLAIM IS:—

1. The new antibiotic aspartocin containing the elements carbon, hydrogen, nitrogen, sulphur and oxygen in substantially the following proportions by weight:

Carbon	53.58
Hydrogen	7.58
Nitrogen	13.58
Sulphur	0.36
Oxygen (by difference) ..	24.90

and having an optical rotation $[\alpha]_D^{25^\circ}$ = +26.4° (c, 2.1% solution in methanol), no characteristic ultraviolet spectrum and when suspended in a potassium bromide pellet exhibiting characteristic absorption in the infra-red region of the spectrum (as indicated in the accompanying drawing) at the following wavelengths expressed in microns: 3.07, 3.45, 6.03, 6.53, 6.89, 7.15, 8.10, and 9.82; and having hydrolysates of the following amino acid contents:

	%	Molar Ratio
D or L-aspartic Acid	35	4
L-Proline ..	8	1
L-Valine	8	1
Glycine	10	2
Glutamic acid ..	10	1

and salts of aspartocin.

2. A process for the production of the new antibiotic aspartocin which comprises cultivating an antibiotic aspartocin producing strain of *Streptomyces griseus* var. *spiralis* or *Streptomyces violaceus* in an aqueous nutrient medium containing assimilable sources of carbon, nitrogen and inorganic salts under submerged aerobic conditions.

3. A process according to Claim 2, in which the cultivation is carried out for a period of from 24 to 240 hours and at a temperature of from 20 to 35° C.

4. A process according to Claim 2 or 3, in which an alkaline earth metal salt such as a calcium salt is added to said medium so that a corresponding salt of aspartocin is produced.

5. A process according to Claim 4, in which aspartocin is recovered from the fermented medium by filtering, and extracting aspartocin from the mycelial cake.

6. A process according to Claim 5, which includes extracting aspartocin from the mycelial cake with water at a *p*H of 1—2 and/or 9—10, solvent extracting the aspartocin from the aqueous extract so produced using a water-immiscible or partially water-immiscible organic solvent, such as butanol, and separating the aspartocin therefrom.

7. A process for the production of the new antibiotic aspartocin substantially as hereinbefore described.

8. The new antibiotic aspartocin whenever produced by the process according to any of Claims 2 to 7.

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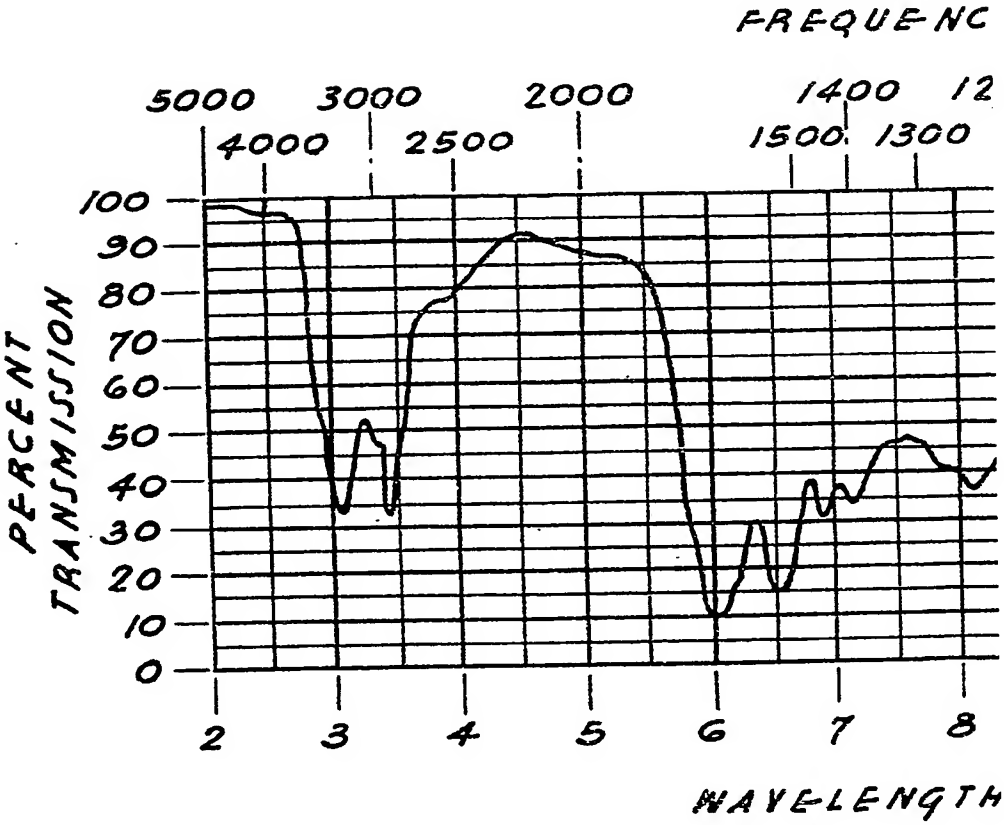

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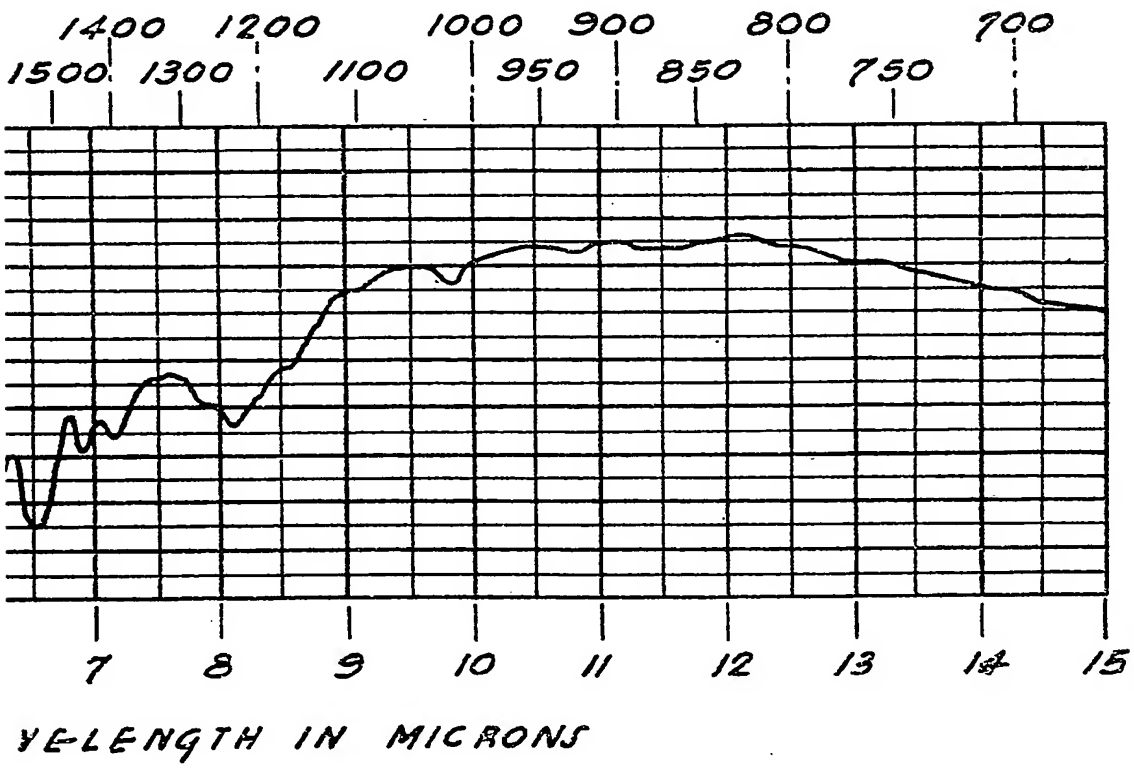
SORPTION SPECTRUM OF ASPARTOCIN



897,581 COMPLETE SPECIFICATION
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